THE COPPER CONTENT AND COPPER TOLERANCE OF SOME SPECIES OF MOLLUSKS OF THE SOUTHERN CALIFORNIA COAST

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This report is mainly concerned with the results of analyses of specimens of some species of marine mollusks with reference to the variation of copper content with age or size, and the approximate upper limits of copper concentration in sea water that a few of them can tolerate.

Some quantitative determinations of the copper content of marine animals have previously been reported, particularly by Hiltner and Wichmann (1919), by Rose and Bodansky (1920), by Severy (1923), and by Galtsoff and Whipple (1931). These investigators found wide variations in the copper concentration of some of the species they studied, and Hiltner and Wichmann (1919) point out that both copper and zinc can be absorbed and retained in the tissues of oysters in much larger quantities than is needed for normal metabolism. Prytherch (1931) obtained evidence that copper is required for the attachment, metamorphosis, and survival of the oyster. Orton (1924) examined the English oyster for this element and mentions the poisonous effects on marine life of excess copper in sea water.

Copper occurs in combination as the chromoprotein hemocyanin in many species of arthropods and mollusks. For a review and bibliography of the work concerning this respiratory pigment, the reader is referred to the paper by Redfield (1934). Elvehjem (1935) has reviewed and discussed the biological significance of copper.

Methods

The applicability of the sodium diethyldithiocarbamate method of Callan and Henderson (1929) to the determination of copper in biological materials has been established by McFarlane (1932) and by Tompsett (1934*a*, *b*, *c*, 1935). In this investigation, the procedure adopted was similar to theirs.

Water, redistilled in an all-Pyrex glass apparatus, was used for preparing reagents and rinsing containers. The reagents employed were tested and found copper-free. Tissues were ashed in silica vessels

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that had previously been treated with sodium acetate. The moist tissue was dried at 105–120°, weighed, and ashed in a muffle furnace at 380–420°. When necessary, small quantities of dilute nitric acid were added to hasten the oxidation of the carbon. A few grams of sodium phosphate were usually mixed with the dry tissue before ashing. The ash was treated with 6 N hydrochloric acid, or first with the 6 N acid, and then with redistilled water. An aliquot portion of solution was transferred to a glass-stoppered graduated cylinder which contained small quantities of sodium pyrophosphate and 25 per cent sodium citrate solutions. Both 4 per cent and saturated solutions of sodium pyrophosphate were used in the course of this work, and the volumes employed depended on the estimated quantities of iron present in the unknowns. The solutions in the cylinders were finally made alkaline to litmus with ammonium hydroxide. The standards contained 0.10 or 0.20 mg. of copper as cupric sulphate besides the other reagents.

After cooling, 1 ml. or 2 ml. of a 2 per cent sodium diethyldithiocarbamate solution was added and the mixture shaken, and then 10 ml. or integral multiples thereof of benzene, toluene, xylene, or ligroin was introduced by pipette for extraction of the colored complex. The whole was then vigorously agitated. It was somewhat difficult to recover the solvent layer completely, or in the same quantities from standard and unknown. Therefore, the same fractional amounts were removed by pipette from each, and the colorimetric determination made. This procedure is correct if the quantities of organic solvent dissolved in the solution containing the standard and that to be tested are the same. Since n-amyl alcohol, which was used by McFarlane (1932), is fairly soluble in water, the other solvents which are much less soluble were substituted for the alcohol. Thus a possible error was avoided. Any water or precipitated material in the solvent layers was removed by centrifuging for a minute or two.

Since a few grains of sand were sometimes found present in the ash, even though considerable effort was made to remove them all from the moist tissues, these may have been a source of error. However, when small samples of sand, taken from the beach where many of the mollusks were obtained, were mixed with sugar charcoal and the above procedure carried out, the quantity of copper found was negligible. Although this is not evidence that such would always be the case, errors thus produced would be expected to be small.

According to Griffiths (1892), manganese is found in the respiratory pigment of the mollusk, *Pinna squamosa*. It is quite probable that this element occurs in other species of mollusks, and it was thus de-

sirable to determine whether or not it could cause discrepancies in the analyses.

It was found that the reddish product formed when manganous chloride or sulphate react with sodium diethyldithiocarbamate is not readily extracted from the aqueous layer by benzene or toluene unless an electrolyte, such as, for example, HCl, NaCl, NH_4OH, is present. However, sodium pyrophosphate was found to form a non-ionizable compound with manganese as it does with iron when the solution is made basic with ammonium hydroxide. An alkaline solution may contain as much as 2.0 mg. of manganese and only .10 mg. of copper, but if sufficient pyrophosphate is present, no appreciable error results in the colorimetric determination for copper.

Zinc, which is also known to be present in mollusks, does not form a colored complex with the carbamate reagent. According to Callan

TABLE I

Copper recovery experiments

Series	Wt. of moist tissue	Quantity of copper added	Total copper found	Copper in tissue
	grams	*mg.*	*mg.*	*mg.*
A	30	0	0.158	0.158
	30	0.020	0.173	0.153
	30	0.060	0.211	0.151
B	30	0	0.071	0.071
	30	0.050	0.102	0.052
	30	0.080	0.139	0.059

and Henderson (1929), this element may be present in as high a concentration as 0.1 gram per 100 ml. and will not interfere with the copper determination provided sufficient ammonia is present and not too much of the colorimetric reagent has been introduced.

Copper Recovery Experiments

Representative data obtained in a series of copper recovery experiments are given in Table I. In each series, the tissues of a number of mussels, *Mytilus californianus*, of various sizes were minced, drained fairly free of fluids, and well mixed. Portions were then weighed out. Copper was introduced by means of a standard cupric sulphate solution containing 0.10 mg. of copper per ml. In the second series of experiments, 3 grams of anhydrous sodium phosphate was added to each vessel. No sodium phosphate was used in the others, but good recoveries resulted.

TABLE II

The copper content of mollusks

Species	Length or weight of animals	Mg. of Cu per 100 grams of moist tissue	Mg. of Cu per 100 grams of dry tissue
Archidoris montereyensis	30.5 grams (one animal)* [a]	0.13	0.59
Astraea undosa (wavy top)		0.80	2.83
		1.26	4.34
Chione undatella (cockle)	2.7–3.9 cm., mean 3.6 cm. (7 animals) [b]	0.12	0.94
		0.20	1.24
Donax gouldii (bean clam)		0.06	0.41
		0.07	0.44
Helix aspersa (common terrestrial snail)	0.1–1.5 grams	1.63	10.7
	4.7–7.6 grams	10.8	56.1
Ischnochiton conspicuus (conspicuous chiton)	22–32.5 grams	0.37	1.03
		0.25	0.97
		0.28	1.08
Navanax inermis (striped mollusk)	51 grams (one animal) [c]	0.44	4.98
Mytilus edulis (bay mussel)		0.23	1.17
		0.37	2.38
Paphia staminea var. *laciniata* (rock cockle)		0.24	1.31
		0.18	1.48
	4.0 ± 0.2 cm.	0.21	1.68
	5.2 ± 0.1 cm. (three animals) [d]	0.18	1.44
Pecten circularis aequisulcatus (speckled scallop)	5.5–6.5 cm. [e]	0.31	2.40
Tegula gallina (speckled turban)		0.61	2.03
		0.61	2.30
Tegula viridula var. *ligulata* (banded turban)		0.81	3.25
		0.92	5.93
Tivela crassatelloides (clam)	[f]	0.21	1.19
		0.23	1.39

* Amount of copper per animal: [a] 0.039 mg.; [b] 0.006 mg.; [c] 0.213 mg.; [d] 0.009 mg.; [e] 0.043 mg.; [f] 0.013 mg.

Results

For a description of the species investigated, refer to Johnson and Snook (1927). Some general data are given in Table II. In this instance, the tissues of from one to twenty animals were usually employed in an analysis without reference to the size of the organisms. Where ranges of dimensions are given, they represent the maximum for the shells. The best basis for comparison of results is that of the dry

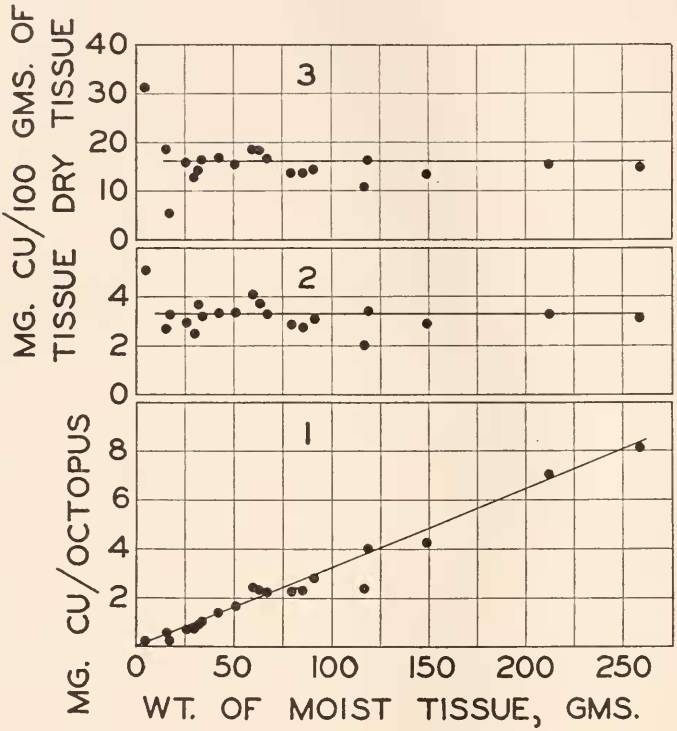

Fig. 1. Variation of copper content with size in the octopus, *Polypus bimaculatus*. Chart 1, mg. of copper per octopus; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

tissue, since it was generally not possible to remove the excess sea water completely from the moist tissues without endangering the loss of body fluids. Even heat-dried tissue may have caused inconsistencies if the animals had different proportional amounts of oxidizable lipoids, fats, and allied substances. This factor was believed to be less serious than those which would have to be reckoned with in the use of wet weights. A vacuum oven was not available. Concentration, in this

case, means the calculated copper content per unit weight of tissue and does not imply a homogeneous distribution.

Severy (1923) found no copper in the Pacific coast clams, *Ensis americanus* and *Venus kennicottii*. However, the writer found copper in small concentrations in both *Donax gouldii* and *Tivela crassatelloides*.

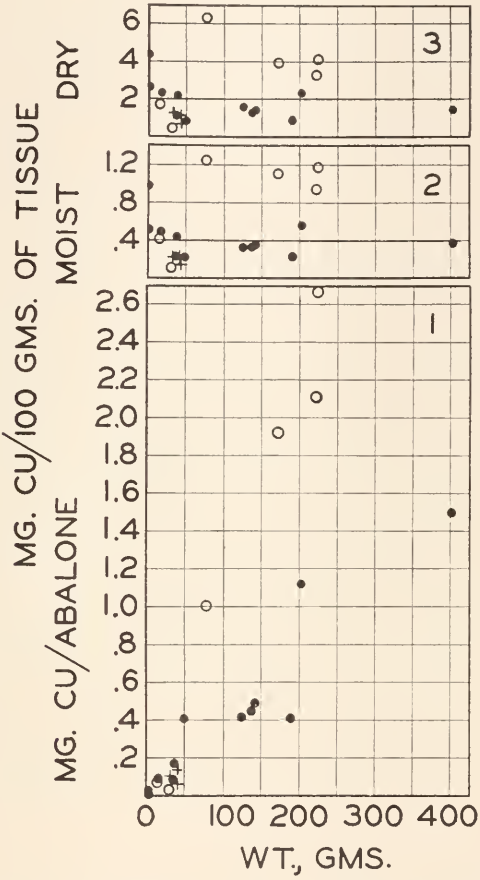

FIG. 2. Variation of copper content with size in the abalones, *Haliotis fulgens*, ○ = *H. cracherodii*, + = *H. rufescens*. The abscissa represents the weight of the moist tissue of each individual. Chart 1, mg. of copper per abalone; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

It was of interest to determine the copper content of animals of different sizes of a few species and to learn whether or not there is any change in the copper content per unit weight of tissue with age or size. Data for six species are plotted in Figs. 1 to 6. Each plotted point

represents the results of an analysis on a single individual with the exception of the data concerning the California sea mussel. External shells were excluded in the analyses.

The copper content in mg., s, of the octopus, *Polypus bimaculatus*,

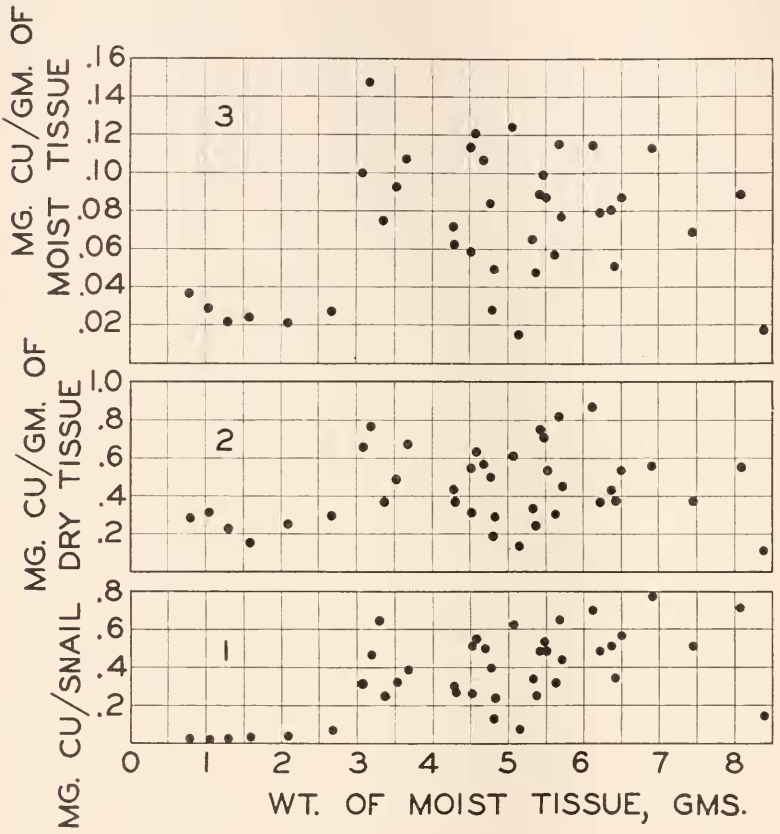

FIG. 3. Variation of copper content with size in the terrestrial snail, *Helix aspersa*. The abscissa represents the weight of the moist tissue of each individual. Chart 1, mg. of copper per snail; Chart 2, mg. of copper per gram of dry tissue; Chart 3, mg. of copper per gram of moist tissue.

varies linearly with the weight in grams, m, of the animal (Fig. 1) and is given by the equation, $s = 0.031\ m$. Mean values of the copper concentration are 3.15 mg. of copper per 100 grams of moist tissue, and 15.6 mg. of copper per 100 grams of dry tissue. The data indicate that these concentrations are constant over the range investigated.

Data concerning the species of abalones, *Haliotis fulgens*, *H. cracherodii*, and *H.* rufescens are plotted in Fig. 2.

Analyses were made of specimens of the terrestrial snail, *Helix aspersa*, for comparative purposes, and results are plotted in Fig. 3. These animals were collected in the late winter and early spring during a rainy period and, for the most part, were not in the resting stage at the time of collection. They were usually kept in the laboratory a few days prior to the analysis. The copper content of individuals which had approximately the same weight was found to be quite variable.

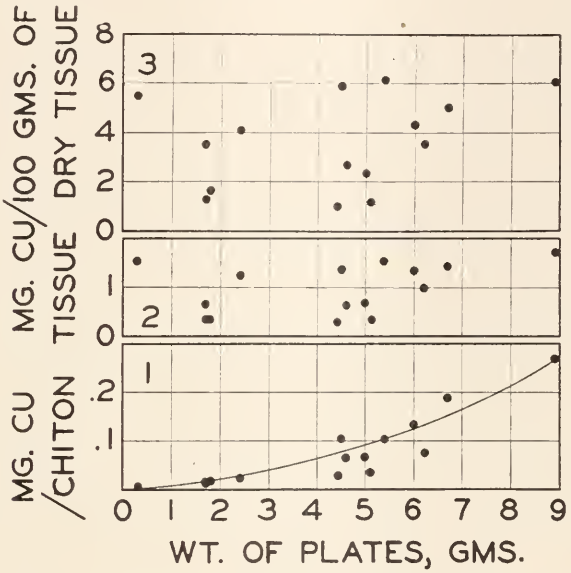

FIG. 4. Variation of copper content with size in the chiton, *Ischnochiton conspicuus*. The abscissa represents the weight of the plates of each individual. Chart 1, mg. of copper per chiton; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

The copper concentration in both moist and dry tissues increases with increase in size of the animals. This fact is also shown by the data in Table II which were obtained by analyzing two groups of animals of a smaller and a larger definite weight-range. Severy (1923), likewise, found a much higher copper concentration in older specimens of the terrestrial mollusk, *Limax maximus*, than in less mature ones. Both the octopus and the snail contain sufficient copper to cause the ash of these animals to be quite blue.

Results of analyses for copper in the chiton, *Ischnochiton conspicuus*, and in the sea hare, *Tethys californica*, are given in Figs. 4 and 5, re-

spectively. It is not known whether or not hemocyanin is the respiratory pigment in these species, whereas it is hemocyanin in the octopus and the snail, but in view of the relatively small amounts of copper in the sea hare it seems rather unlikely in this instance. Because of the difficulty of completely removing excess sea water from the chitons, the weight of the plates of each animal has been used as a measure of size.

The copper concentration in the dry tissue of the California sea

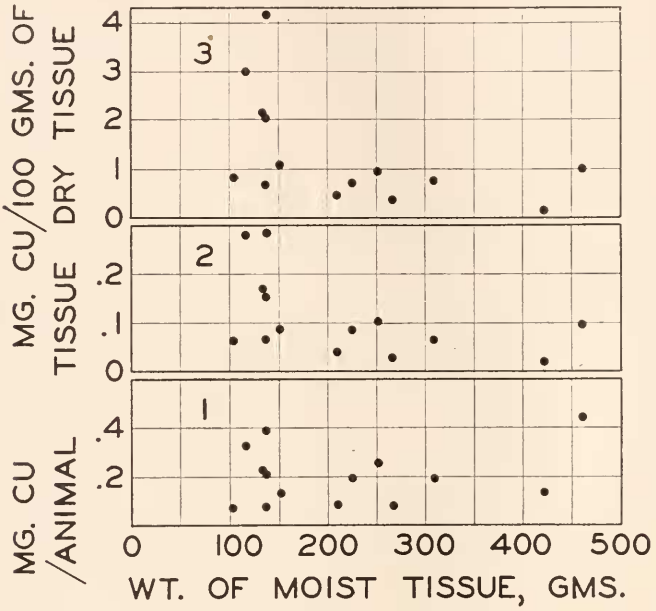

FIG. 5. Variation of copper content with size in the sea hare, *Tethys californica*. The abscissa represents the weight of each animal. Chart 1, mg. of copper per sea hare; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

mussel falls off with increase in length (Fig. 6) of the valves. In general a number of animals were used in each analysis, and these were selected so that they had the same length within ± 0.5 cm. of the mean for the larger sizes and ± 0.2 cm. for the smaller sizes. Selection was made by using length of valves rather than the weight of the moist tissue because the procedure was then more rapid and more accurate. The weight of the moist tissue cannot be determined with the same accuracy as the length of the valves because of the accompanying sea water.

An investigation was made of the variation of copper content and concentration in different groups of organs obtained from young mussels and more mature ones, and data are given in Table III. The tissues of these animals were first hardened in a 2 per cent formaldehyde solution before removal from the valves. Since both the gonads and mantle contain eggs or sperm, these organs were grouped together. The foot is largely muscular tissue. No attempt was made to separate males from females. The decrease in copper concentration with increase in size is to be noted.

Chou and Adolf (1935) examined certain organs of five cadavers for

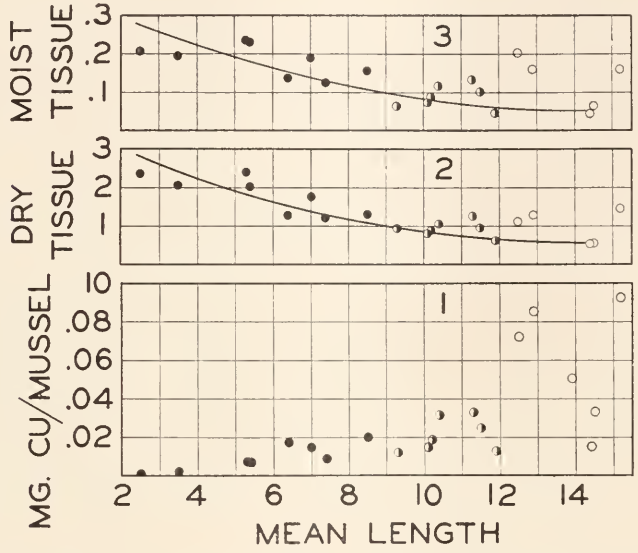

Fig. 6. Variation of copper content with size in the California sea mussel, *Mytilus californianus*. The abscissa represents the mean length of the valves when a number of animals were used. ○, one animal; ◑, 2–3 animals; ●, 5 or more animals. Chart 1, mg. of copper per mussel (mean values); Chart 2, mg. of copper per 100 grams of dry tissue; Chart 3, mg. of copper per 100 grams of moist tissue.

copper and found a markedly higher concentration in the infants than in the adults. Sheldon and Ramage (1931) analyzed five foetal livers of man for copper and found a concentration 5 to 7 times that in adult organs. Perhaps copper plays a significant rôle in growth metabolism in many animals.

Copper Content of Sea Water

In connection with the work concerning the copper tolerance of certain mollusks, it was desirable to know the copper content of the ocean water in which they live.

Using a procedure similar to that of Atkins (1933), determinations were made of the copper concentration in surface water taken 1,000 feet from shore at this station. The water was collected in a glass bucket and stored in Pyrex containers. Cursory examinations, made by adding 1 ml. of 2 per cent sodium diethyldithiocarbamate solution to 1 liter of sea water, extracting with 10 ml. of carbon tetrachloride

TABLE III

The copper content of groups of organs of the sea mussel, *Mytilus californianus* *

Length of mussels	No. of mussels used	Hepatopancreas, intestines and heart	Mantle and gonads	Muscles and foot	Gills and palps
cm.					
Range: 6.3–7.5	30	(*a*) 0.0038	0.0032	0.0022	0.0047
Mean: 6.9		(*b*) 27.4	23.0	15.8	33.8
		(*c*) 1.34	0.20	0.36	0.65
		(*d*) 9.35	2.52	2.26	7.70
Range: 6.6–7.7	30	(*a*) 0.0049	0.0034	0.0025	0.0052
Mean: 7.1		(*b*) 30.6	21.2	15.6	32.6
		(*c*) 1.66	0.20	0.36	0.55
		(*d*) 13.6	2.79	2.44	7.07
Range: 11.9–13.0	5	(*a*) 0.0159	0.0216	0.0042	0.0148
Mean: 12.5		(*b*) 28.2	38.2	7.4	26.2
		(*c*) 0.86	0.21	0.14	0.39
		(*d*) 8.70	3.26	1.00	5.44
Range: 11.8–12.4	4	(*a*) 0.0172	0.0234	0.0039	0.0102
Mean: 12.1		(*b*) 31.5	42.8	7.1	18.6
		(*c*) 0.85	0.23	0.12	0.27
		(*d*) 8.21	1.65	0.57	3.66

* The data for each group of organs of each series is placed in the following order:
(*a*) mg. of copper per group of organs per mussel (mean values),
(*b*) per cent of total copper present per mussel,
(*c*) mg. of copper per 100 grams of moist tissue,
(*d*) mg. of copper per 100 grams of dry tissue.

or benzene and estimating colorimetrically, showed that the copper present could not be greater than 0.004 mg. per kg.

Twenty liters were electrolyzed, 1 liter at a time, between platinum electrodes at a potential difference of approximately 2 volts for at least 8 hours, and at a temperature of 70–90° during much of this period. The copper was dissolved from the cathode with dilute copper-free nitric acid, the solution was then neutralized with ammonium hydroxide, cooled, 1 ml. of the carbamate reagent introduced and the total extracted with benzene. A colorimetric determination showed

0.0010 mg. of copper present per kg. of sea water (salinity 33.68 per cent). An analysis of a 10-liter sample taken six weeks later showed 0.0014 mg. of copper present per kg. (salinity 33.63 per cent). There had been no rain in this region for several months.

Copper Tolerance of a Few Species of Marine Mollusks

The approximate upper limits of copper concentration that some mollusks can tolerate, when kept in the laboratory under conditions to be described, were determined as follows.

Table IV

The copper tolerance of marine mollusks

Mg. of copper added per kg. of sea water		Time of survival, days				
		0	0.05	0.10	0.15	0.20
Species	Animals per jar					
Acmaea scabra var. *limatula*	4	16†		3†		3†
Fusinus kobelti	2–3	60		60		8†
Astraea undosa	2	30	14†	5†		5†
Haliotis fulgens	1–2	30	30	3†		
Ischnochiton conspicuus	2–3	60		60	10†	3†
Mytilus californianus	2	60		60	30	2†
Mytilus edulis	3	35		35		17†
Tegula gallina	3–4	60	60	15†		8†
Tegula viridula var. *ligulata*	4	60	60	25†		18†
Paphia staminea var. *laciniata*	3–4	100 alive at end of 30 days when 1.0 mg. of Cu added per kg. One animal alive at end of 65 days when 3.0 mg. of Cu added per kg.; another lived 58 days in latter concentration.				

† Dead at end of this time. All others alive at end of stated period.

In a series of jars, each of which contained 8 kg. of sea water, were placed a number of mollusks of a given species. Definite amounts of a standard cupric chloride solution containing 0.08 mg. of copper per ml. were then added to each jar. A continuous stream of air was passed in to keep the water well aerated, and distilled water added every day or two so that the volume and salinity were maintained constant. Many of the experiments were run in duplicate. Controls, to which no copper had been added, were run simultaneously. Temperature variations corresponded approximately to those of the sea. In general,

there was a growth of algae, bacteria, etc. so that the animals were not without food. However, it should be pointed out that the natural habitat was not completely duplicated and other factors may have contributed to a decrease of the life span, such as the effect of added copper on the growth of certain organisms used for food.

Results are presented in Table IV. The upper limit of copper tolerance for most of the species investigated lies in a range of 0.10 to 0.20 mg. of added copper per kilogram of sea water and thus is about 100 to 200 times that normally present. However, the clam, *Paphia staminea* var. *laciniata*, can tolerate a much higher concentration. The addition of 3.0 mg. of copper as cupric chloride to a kilogram of sea water produces a noticeable cloudiness. Further introduction of copper alters the concentration of some of the other constituents because of precipitation.

Since younger mussels have a higher copper content per unit weight of tissue than more mature ones, it was thought that the former might tolerate 0.20 mg. of added copper per kilogram, but this was found to be untrue. The valves of the animals in this instance had a mean length of 5.3 cm.

Summary

The copper content and concentration of several species of mollusks has been determined with particular reference to variation with age or size. In the snail, *Helix aspersa*, the amount of copper per unit weight of tissue increases with increase in size, whereas it decreases in the California sea mussel. In the octopus, *Polypus bimaculatus*, it remains constant. Samples of sea water of the Pacific were found to contain 0.001 mg. of copper per kilogram. The upper limits of copper tolerance of a few species of marine mollusks, when kept in the laboratory under given conditions, lies in the range from 0.10 to 0.20 mg. of added copper per kilogram of sea water. The clam, *Paphia staminea* var. *laciniata*, can tolerate a much higher concentration than the above.

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